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Induction of Mutation in *Streptococcus diacetilactis* by Nitrosoguanidine and Ultraviolet Irradiation [1]

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Abstract

A culture of *Streptococcus diacetilactis* (DRC_3) was exposed to the mutagenic action of N-methyl-N′-nitro-N-nitrosoguanidine and ultraviolet irradiation, singly and in combination. There was considerable variation in total acidity and production of flavor compounds for cultures, irrespective of the mutagenic treatment, indicating possible genetic changes in exposed cells. An average of 0.3% titratable acidity was obtained for mutants in contrast to 0.45% for the untreated culture. Eight mutants obtained after combined treatment produced higher amounts of diacetyl. One of the mutants (M_1) did not coagulate milk up to 48 hours compared to the performance of other isolates and the parent culture. The capacity to produce acid was partially impaired in this mutant strain. There were no significant differences between it and the parent culture in production of volatile acidity, diacetyl, acetoin, and proteolytic activity. However, mutant strain M_1 produced nearly double the amount of acetaldehyde.

Introduction

Although there have been several reports on the mutagenic action of N-methyl-N′-nitro-N-nitrosoguanidine (NTG) on different types of microorganisms (1, 6, 11, 12, 16), there is very little information on the use of this compound for lactic acid bacteria except in a recent report by Burrow et al. (3). In our study a strain of *Streptococcus diacetilactis* (DRC_3) was exposed to mutagenic action of NTG and ultraviolet irradiation, singly and in combination, with the object of obtaining possible genetic variants with increased capacity to produce flavor compounds.

Experimental Procedures

A strain of *Streptococcus diacetilactis* (DRC_3) maintained in the culture collection of our National Dairy Research Institute was selected.

Treatment with N-methyl-N′-nitro-N-nitrosoguanidine. A nine-hour old culture of *S. diacetilactis* grown in yeast dextrose broth was distributed equally into several test tubes of uniform diameter. The cell concentration of the original suspension was adjusted between 0.55 to 0.66 OD in a Hilger's Absorptiometer. Nitrosoguanidine was added to different tubes from 10 to 500 μg per milliliter and the contents mixed well. One tube was the control. The mutagen acted for one hour on the agitated cell suspension. Treated as well as control cell suspensions were centrifuged and the cells washed with 0.02 M phosphate buffer (pH 7.0). The washed cells were resuspended in yeast dextrose broth and incubated for one hour at 30 C. After re-centrifuging and washing, the cells were suspended in phosphate buffer. Appropriate dilutions were plated on citrate agar (15) and incubated at 30 C for 48 hours. Percentage of survivors was calculated by counting colonies in the treated and untreated control plates. The petri plates showing less than 50% survival (1) as compared to the control were selected for isolating individual colonies.

Treatment with ultraviolet irradiation. Twenty milliliters of the untreated washed cell suspension in the aforementioned buffer were exposed to ultraviolet irradiation for different times. A 15-w germicidal lamp in a specially constructed wooden cabinet was the radiation source. During irradiation, the suspension was stirred magnetically. The dosage of ultraviolet irradiation was calculated from time of exposure and the intensity of ultraviolet light as measured in UV doze-rate meter (M/s Ultraviolet Products, California, U.S.). At intervals aliquots of the irradiated cell suspension were withdrawn from the petri dish and dilutions were plated on citrate agar. The irradiation experiments were performed in the dark. Individual colonies were isolated from plates showing 1.0 to 0.1% survivors.

Treatment with nitrosoguanidine followed by ultraviolet irradiation. Twenty-milliliter aliquots each of NTG-treated (250 and 500 μg/ml) cell suspensions in phosphate buffer were exposed separately to ultraviolet irradiation as

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described. Petri plates showing 1.0 to 0.1% survival after irradiation were selected for isolating colonies.

Examination of survivors for variation in characteristics. The isolates of survivors picked from different plates were subcultured in reconstituted nonfat milk and compared with the parent culture for genetic variations in acid and flavor production. The cultures were grown in sterile reconstituted nonfat milk at 30 C and examined for titratable acidity at six hours and for diacetyl and acetoin production at 18 hours.

Detailed study of a selected mutant (M_1). One of the mutants (M_1), recovered after combined mutagenic treatment, did not curdle milk up to 48 hours in contrast to that of other isolates and the parent strain. This strain was characterized for morphological, cultural and biochemical characteristics to establish its taxonomic identity with the parent culture. The mutant was also examined for its sensitivity to *S. diacetilactis* (DRC_3) phage by the plaque technique.

The mutant was also compared with the parent culture for rate of growth, development of total titratable and volatile acidity, production of diacetyl, acetoin, acetaldehyde and proteolytic activity.

Methods of analysis. Titratable acidity was determined by titrating a known aliquot of the sample against 0.1 N NaOH. Determinations of volatile acidity, diacetyl, acetoin and acetaldehyde were performed by the methods of Hempenius and Liska (8), Pack et al. (17), Anderson and Leesment (2) and Lindsay and Day (13). Proteolytic activity of the cultures was estimated by the method of Hull (9).

Taxonomic characteristics were according to Manual of Microbiological Methods (14).

The total cell counts (direct microscopic count) and viable cell counts (standard plate count) of the cultures were determined according to standard procedures (10).

Results and Discussion

Survival of cells after treatment. The percentage of survivors after exposing the cells of *S. diacetilactis* (DRC_3) to different doses of ultraviolet irradiation is shown in Figure 1(a). There was a progressive and rapid destruction

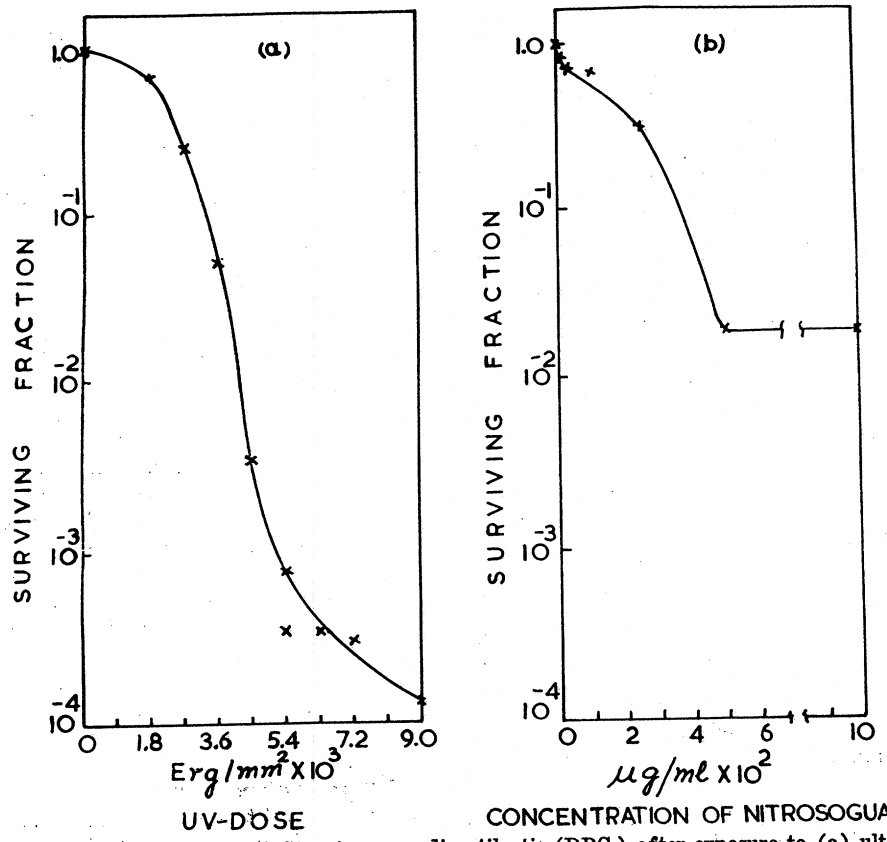

FIG. 1. Survival curves of *Streptococcus diacetilactis* (DRC_3) after exposure to (a) ultraviolet irradiation and (b) nitrosoguanidine.

of cells when ultraviolet dosages were increased from 2,700 to 5,400 ergs per square millimeter.

Data regarding survival of cells after exposure to nitrosoguanidine represented in Figure 1(b) indicate that approximately 50% survival was obtained when the cells were exposed to 250 μg per milliliter of NTG. Treatment of cells with double the concentration of NTG (500 μg/ml) resulted in nearly 98% destruction. Increasing mutagen to 1,000 μg per milliliter did not significantly change survival rate.

Variation in characteristics of survivors. Attempts to develop a rapid screening method for detecting high diacetyl-producing variants by measuring the diameter of the clear zones around individual colonies on citrate agar were not successful as it was not possible to correlate the diameter of the zone with diacetyl production. Similarly, the impression method adopted by Grinevich (7) for the quick screening of variants showing increased diacetyl production did not give satisfactory results. As the estimation of diacetyl involved a laborious process, only a limited number of isolates of survivors after each type of mutagenic treatment could be examined for acid and flavor production and these data are presented in Table 1.

There was considerable variation in total acidity and production of flavor compounds in many cultures of survivors irrespective of the mutagenic treatment, indicating possible genetic changes in the exposed cells. It is of interest that the survivors after exposure of cells to 500 μg/ml of NTG followed by ultraviolet irradiation, markedly decreased acid production. An average of 0.30% titratable acidity was obtained in the mutants in contrast to 0.45% produced by untreated parent culture (Table 1). Nitrosoguanidine and ultraviolet-treated cultures, either singly or in combination, decreased acetoin and diacetyl production. However, in eight mutants obtained after combined treatment, diacetyl production was measurably higher in contrast to that for the parent culture. A few typical mutant cultures produced eiher very small amounts of acetoin or diacetyl or had totally lost the ability to produce one or the other of the flavor compounds. One of of the mutants recovered after ultraviolet irradiation failed to produce both diacetyl and acetoin. Similar results have been reported by Burrow et al. (3), whereas Collins and Harvey (5) isolated natural variants which failed to produce acetoin and diacetyl.

Detailed study of the mutant strain (M_1). Mutant strain M_1 formed very long chains up to 18 cells whereas the parent culture had mostly diplococci, occasionally in short chains. When verified for sensitivity to bacteriophage, both the parent and mutant (M_1) cultures were sensitive to the same *S. diacetilactis* (DRC_3) phage, thereby indicating that the mutant has been derived from the parent culture.

Mutant M_1 did not coagulate milk up to 48 hours compared to the performance of the parent culture which coagulated milk within 18 hours. A slow acid producing natural variant of *S. lactis* which required at least 48 hours to coagulate milk, has been reported by Citti et al. (4). The mutant produced 60% less acid after 24 hours as compared to the parent culture. It is of interest that the rate of growth of the mutant culture was similar to that of the parent up to nine hours of incubation and thereafter the parent culture slowed down presumably due to the production of larger amount of acid. The ratio between total cell count and clump counts

TABLE 1. Acid and flavor production by mutants of *Streptococcus diacetilactis* (DRC_3) obtained after exposing the cells to mutagenic agents.

Treatment	Survivors examined		Titratable[a] acidity	Acetoin[b]	Diacetyl[b]
	(no.)		(%)	(ppm)	(ppm)
Untreated	Parent culture		0.45	140.0	30.0
NTG	20	Avg	0.42	92.0	14.0
		Range	0.35–0.55	0.0–175.0	6.0–23.0
UV	30	Avg	0.38	91.0	13.0
		Range	0.27–0.44	0.0–180.0	0.0–24.0
NTG + UV	20	Avg	0.39	73.0	11.0
(250 μg/ml)		Range	0.34–0.42	38.0–138.0	0.0–21.0
NTG + UV	30	Avg	0.30	102.0	27.0
(500 μg/ml)		Range	0.18–0.40	55.0–161.0	0.0–54.0

[a] Determined after six hours of incubation at 30 C in reconstituted nonfat milk.
[b] Determined after 18 hours of incubation at 30 C in reconstituted nonfat milk.

TABLE 2. Comparison of acid and flavor production and proteolytic activity of mutant (M_1) and parent culture of *Streptococcus diacetilactis*.[a]

	Parent	Mutant (M_1)
Titratable acidity (Per cent lactic)	1.07	0.50
Volatile acidity (Ml of 0.01 N NaOH/ 50 g of curd)	25.0	20.5
Acetyl methyl carbinol (Ppm)	140.0	135.0
Diacetyl (Ppm)	30.0	35.0
Acetaldehyde (Ppm)	4.0	7.5
Proteolytic activity (Mg of tyrosine liberated/ g of curd)	0.60	0.53

[a] Cultures grown in reconstituted nonfat milk and examined after incubation at 30 C for 18 hours.

ranged from 2.2 to 4.7 for the mutant, whereas in the parent it varied from 1.5 to 2.2. No significant differences were noted in the viable counts of the two cultures at the end of 24 hours.

Mutant (M_1) and the parent strains did not show any significant differences in production of volatile acids, diacetyl and acetoin and proteolytic activity. However, the acetaldehyde production in the mutant culture (M_1) was nearly double that of the parent culture (Table 2). The mutant strain did not show any further physiological variations even after prolonged maintenance and repeated subculturing for six months. Mutants capable of producing significant amounts of acetaldehyde have recently been isolated by Burrow et al. (3) from a culture of *S. diacetilactis* although they were unable to produce any diacetyl.

Acknowledgment

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